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APPLICATION

for

UNITED STATES LETTERS PATENT

To all whom it may concern:

Be it known that ***MARTIN WYBE KAST,***
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has(ve) invented certain new and useful improvements in

PEPTIDES OF HUMAN PAPILLOMA VIRUS FOR USE IN HUMAN T CELL RESPONSE

of which the following is a full and exact description.

Peptides of Human Papilloma Virus for use in human T cell response inducing compositions

Field of the invention

The invention is concerned with novel peptides derived from Human Papilloma Virus proteins and their use in pharmaceutical compositions for a prophylactic or therapeutic treatment of human individuals against Human Papilloma Virus-related diseases such as cervical cancer.

Background of the invention

Human Papilloma Viruses (HPVs) are implicated in the etiology of cervical cancer, the fifth most common cancer worldwide and the second cause of cancer-related death in women. If also other HPV-related cancers are taken into acccount, up to 10% of the worldwide mortality due to cancer is linked to HPVs. HPVs are double stranded circular DNA viruses of about 8 kilobases. Until now more than 60 genotypes have been described of which several are associated with cancer.

HPV-DNA can be found in cervical dysplastic lesions and in cervical carcinomas in which the percentage of HPV positivity increases up to 99% when the lesions progress towards malignancy. The most important HPV types associated with cervical carcinoma are HPV16 and 18 of which HPV16 alone accounts for more than 50% of the HPV positive cervical carcinomas.

The DNAs of several HPVs have been sequenced. The DNA open reading frames can be divided into early regions (E) and late regions (L). The E regions are coding for proteins needed for virus replication and transformation. The L regions encode viral capsid proteins. The E6 and E7 proteins are involved in the pathogenesis of HPV-induced abnormal cell proliferation and these

genes are expressed in tissue or tumor cells obtained from cervical cancers associated with HPV infection.

In addition, the E6 and E7 genes of HPV16 and HPV18 are capable of inducing epithelial cell transformation in the cell culture without the presence of other HPV genes indicating that at least part of the stimulation of cell proliferation caused by HPV infection is due to the E6 and E7 viral proteins.

Cytotoxic T lymphocytes (CTL) are of crucial importance in the resistance against virus infections and the immune surveillance against virus-induced tumors (reviewed by Kast and Melief, 1991). CTL specific for viruses or virus-induced tumors recognize short viral protein-derived peptides, of about 9 amino acids in length, that are bound to the antigen presenting groove of major histocompatibility complex (MHC) class I molecules (reviewed by Kast and Melief, 1991). Recently, in several virus systems vaccination with peptides recognized by antigen-specific CTL was shown to prevent lethal virus infections and to delay tumor growth in mice (reviewed by Kast and Melief, 1991, and by Reinholdsson-Ljunggren et al., 1992).

We have succeeded in the identification of viral peptides that bind to the groove of MHC class I molecules by using the antigen processing defective cell line 174CEM.T2 generated and provided by P. Cresswell (see Salter and Cresswell, 1986). This cell line expresses the human MHC class I HLA-A2.1 and HLA-B5 alleles of which only the HLA-A2.1 molecules are expressed as partly empty and unstable molecules that can be stabilized on the cell surface with exogenously added peptides. If incubation with peptide results in an increase in the cell surface expression of this MHC molecule, this implies that the peptide binds to the groove of the HLA-A2.1 molecule and is therefore a possible candidate to be recognized by CTL. The HLA-A2.1 molecule is the most frequent HLA molecule present in the Western European Caucasoid population. About 50% of this population expresses this allele.

Using the amino acid sequence of the E6 and E7 proteins of HPV16 and HPV18 (Seedorf et al., 1985) we generated all possible

nonapeptides (i.e. 9 amino acid long peptides) overspanning the entire E6 and E7 region. Every amino acid was used as a start amino acid for these 9-mer peptides. Every peptide was subjected individually to the above test to determine its capacity to bind to the HLA-A2.1 molecule. With respect to HPV16, we identified in total 10 peptides in the HPV16 E6 region and 8 in the HPV16 E7 region which bound to the HLA-A2.1 molecule in the above test. With respect to HPV18, in total 9 peptides in the HPV18 E6 region and 5 in the HPV18 E7 region were identified in the above test to bind to the HLA-A2.1 molecule. This implies that important candidate peptides of HPV16 and HPV18 for use as a vaccine in HLA-A2.1 positive humans have been identified.

By using a second approach, we succeeded to expand the list of HLA-A2.1 binding HPV peptides a little further and to determine HPV16 E6 and E7 peptides binding to other HLA molecules, viz. to the HLA-A1, HLA-A3.2, HLA-A11.2 and HLA-A24 molecules. Said second approach consisted of a competitive immunochemical peptide-MHC binding assay using purified class I molecules and radiolabeled consensus peptides.

Summary of the invention

An object of the present invention is to provide synthetic peptides which can be used for prevention, prophylaxis, therapy and treatment of cervical carcinoma and/or adenoma and other HPV-related, in particular HPV16- and/or HPV18-related diseases.

Another object of the invention is to provide a method of prophylactic or therapeutic treatment of cervical carcinoma and/or adenoma and other HPV-related, in particular HPV16- and/or HPV18-related diseases.

A further object of the present invention is to provide pharmaceutical compositions which can be used for prevention, prophylaxis, therapy and treatment of cervical carcinoma and/or adenoma and other HPV-related, in particular HPV16- and/or HPV18-related diseases.

This invention provides a peptide comprising an amino acid sequence derived from a protein of human papilloma virus (HPV), wherein said amino acid sequence has the ability to bind to a human Major Histocompatibility Complex (MHC) Class I molecule.

The present invention also provides specific peptides derived from the amino acid sequence of the E6 and E7 regions of HPV16 and HPV18 which, because of their capability to bind to HLA molecules, such as e.g. the HLA-A2.1, HLA-A1, HLA-A3.2, HLA-A11.2 or HLA-A24 protein, are candidate peptides to be included in human vaccines that can induce protective or therapeutic T cell responses against HPV16 and/or HPV18.

The novel peptides of the present invention are useful in pharmaceutical compositions, as screening tools and in the prevention, prophylaxis, therapy and treatment of HPV16- and/or HPV18-induced diseases or other conditions which would benefit from inhibition of HPV16 and/or HPV18 infection.

In a preferred embodiment of the invention, said amino acid sequence is derived from protein E6 or E7 of HPV16. In another preferred embodiment of the invention, said amino acid sequence is derived from protein E6 or E7 of HPV18.

Preferably, said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1.

More specifically, this invention provides a peptide comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1 and is selected from the group consisting of:

AMFQDPQER	(residues	7- 15 of HPV16 protein E6)
KLPQLCTEL	(residues	18- 26 of HPV16 protein E6)
QLCTELQTT	(residues	21- 29 of HPV16 protein E6)
LCTELQTTI	(residues	22- 30 of HPV16 protein E6)
ELQTTIHDI	(residues	25- 33 of HPV16 protein E6)
LQTTIHDII	(residues	26- 34 of HPV16 protein E6)
TIHDIILEC	(residues	29- 37 of HPV16 protein E6)
IHDIILECV	(residues	30- 38 of HPV16 protein E6)
CVYCKQQLL	(residues	37- 45 of HPV16 protein E6)

FAFRDLCIV (residues 52- 60 of HPV16 protein E6)
KISEYRHYC (residues 79- 87 of HPV16 protein E6)
PLCDLLIRC (residues 102-110 of HPV16 protein E6)
TLHEYMLDL (residues 7- 15 of HPV16 protein E7)
YMLDLQPET (residues 11- 19 of HPV16 protein E7)
MLDLQPETT (residues 12- 20 of HPV16 protein E7)
RLCVQSTHV (residues 66- 74 of HPV16 protein E7)
TLEDLLMGT (residues 78- 86 of HPV16 protein E7)
LLMGTLGIV (residues 82- 90 of HPV16 protein E7)
GTLGIVCPI (residues 85- 93 of HPV16 protein E7)
TLGIVCPIC (residues 86- 94 of HPV16 protein E7), and

a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of these amino acid sequences which has the ability to bind to human MHC Class I allele HLA-A2.1.

More specifically, this invention provides a peptide comprising an amino acid sequence derived from protein E6 or E7 of HPV18, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1 and is selected from the group consisting of:

KLPDLCTEL (residues 13- 21 of HPV18 protein E6)
SLQDIEITC (residues 24- 32 of HPV18 protein E6)
LQDIEITCV (residues 25- 33 of HPV18 protein E6)
EITCVYCKT (residues 29- 37 of HPV18 protein E6)
KTVLELTEV (residues 36- 44 of HPV18 protein E6)
ELTEVFEFA (residues 40- 48 of HPV18 protein E6)
FAFKDLFVV (residues 47- 55 of HPV18 protein E6)
DTLEKLTNT (residues 88- 96 of HPV18 protein E6)
LTNTGLYNL (residues 93-101 of HPV18 protein E6)
TLQDIVLHL (residues 7- 15 of HPV18 protein E7)
FQQLFLNTL (residues 86- 94 of HPV18 protein E7)
QLFLNTLSF (residues 88- 96 of HPV18 protein E7)
LFLNTLSFV (residues 89- 97 of HPV18 protein E7)
LSFVCPWCA (residues 94-102 of HPV18 protein E7), and

a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of these amino acid sequences

which has the ability to bind to human MHC Class I allele HLA-A2.1.

According to another preferred embodiment of this invention, said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A1.

More specifically, this invention provides a peptide comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A1 and is selected from the group consisting of:

YRDGNPYAV	(residues 61- 69 of HPV16 protein E6)
WTGRCMSCC	(residues 139-147 of HPV16 protein E6)
MSCCRSSRT	(residues 144-152 of HPV16 protein E6)
TTDLYCYEQ	(residues 19- 27 of HPV16 protein E7)
EIDGPAGQA	(residues 37- 45 of HPV16 protein E7)
HVDIRTLED	(residues 73- 81 of HPV16 protein E7), and

a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of these amino acid sequences which has the ability to bind to human MHC Class I allele HLA-A1.

According to another preferred embodiment of this invention, said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A3.2.

More specifically, this invention provides a peptide comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A3.2 and is selected from the group consisting of:

AMFQDPQER	(residues 7- 15 of HPV16 protein E6)
IILECVYCK	(residues 33- 41 of HPV16 protein E6)
CVYCKQQLL	(residues 37- 45 of HPV16 protein E6)
VYCKQQLLR	(residues 38- 46 of HPV16 protein E6)
QQLLRREVY	(residues 42- 50 of HPV16 protein E6)
IVYRDGNPY	(residues 59- 67 of HPV16 protein E6)
YAVCDKCLK	(residues 67- 75 of HPV16 protein E6)
AVCDKCLKF	(residues 68- 76 of HPV16 protein E6)
VCDKCLKFY	(residues 69- 77 of HPV16 protein E6)

KFYSKISEY (residues 75- 83 of HPV16 protein E6)
KISEYRHYC (residues 79- 87 of HPV16 protein E6)
ISEYRHYCY (residues 80- 88 of HPV16 protein E6)
RHYCYSLYG (residues 84- 92 of HPV16 protein E6)
SLYGTTLEQ (residues 89- 97 of HPV16 protein E6)
TTLEQQYNK (residues 93-101 of HPV16 protein E6)
QQYNKPLCD (residues 97-105 of HPV16 protein E6)
LIRCINCQK (residues 107-115 of HPV16 protein E6)
HLDKKQRFH (residues 125-133 of HPV16 protein E6)
CMSCCRSSR (residues 143-151 of HPV16 protein E6)
SCCRSSRTR (residues 145-153 of HPV16 protein E6)
CCRSSRTRR (residues 146-154 of HPV16 protein E6)
HYNIVTFCC (residues 51- 59 of HPV16 protein E7)
YNIVTFCCK (residues 52- 60 of HPV16 protein E7)
CCKCDSTLR (residues 58- 66 of HPV16 protein E7)
KCDSTLRLC (residues 60- 68 of HPV16 protein E7), and

a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of these amino acid sequences which has the ability to bind to human MHC Class I allele HLA-A3.2.

According to another preferred embodiment of this invention, said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A11.2.

More specifically, this invention provides a peptide comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A11.2 and is selected from the group consisting of:

AMFQDPQER (residues 7- 15 of HPV16 protein E6)
IILECVYCK (residues 33- 41 of HPV16 protein E6)
CVYCKQQLL (residues 37- 45 of HPV16 protein E6)
VYCKQQLLR (residues 38- 46 of HPV16 protein E6)
QQLLRREVY (residues 42- 50 of HPV16 protein E6)
IVYRDGNPY (residues 59- 67 of HPV16 protein E6)
YAVCDKCLK (residues 67- 75 of HPV16 protein E6)
AVCDKCLKF (residues 68- 76 of HPV16 protein E6)

VCDKCLKFY (residues 69- 77 of HPV16 protein E6)
KISEYRHYC (residues 79- 87 of HPV16 protein E6)
ISEYRHYCY (residues 80- 88 of HPV16 protein E6)
LIRCINCQK (residues 107-115 of HPV16 protein E6)
TGRCMSCCR (residues 140-148 of HPV16 protein E6)
CMSCCRSSR (residues 143-151 of HPV16 protein E6)
SCCRSSRTR (residues 145-153 of HPV16 protein E6)
HYNIVTFCC (residues 51- 59 of HPV16 protein E7)
YNIVTFCCK (residues 52- 60 of HPV16 protein E7)
CCKCDSTLR (residues 58- 66 of HPV16 protein E7)
VCPICSQKP (residues 90- 98 of HPV16 protein E7), and

a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of these amino acid sequences which has the ability to bind to human MHC Class I allele HLA-A11.2.

According to another preferred embodiment of this invention, said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A24.

More specifically, this invention provides a peptide comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A24 and is selected from the group consisting of:

MHQKRTAMF (residues 1- 9 of HPV16 protein E6)
LQTTIHDII (residues 26- 34 of HPV16 protein E6)
VYCKQQLLR (residues 38- 46 of HPV16 protein E6)
LLRREVYDF (residues 44- 52 of HPV16 protein E6)
VYDFAFRDL (residues 49- 57 of HPV16 protein E6)
PYAVCDKCL (residues 66- 74 of HPV16 protein E6)
KCLKFYSKI (residues 72- 80 of HPV16 protein E6)
EYRHYCYSL (residues 82- 90 of HPV16 protein E6)
HYCYSLYGT (residues 85- 93 of HPV16 protein E6)
CYSLYGTTL (residues 87- 95 of HPV16 protein E6)
RFHNIRGRW (residues 131-139 of HPV16 protein E6)
RAHYNIVTF (residues 49- 57 of HPV16 protein E7), and

a fragment, homolog, isoform, derivative, genetic variant

or conservative variant of any one of these amino acid sequences which has the ability to bind to human MHC Class I allele HLA-A24.

This invention further provides a pharmaceutical composition containing a prophylactically or therapeutically effective amount of a peptide according to the invention, and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. Preferably, said pharmaceutical composition contains a peptide according to the invention which is able to induce a T cell response effective against HPV, in particular a HLA class I-restricted $CD8^+$ cytotoxic T cell response.

In addition, this invention provides a method of prophylactic or therapeutic treatment of cervical carcinoma and other HPV-related diseases with a human individual, comprising administering to said human individual a prophylactically or therapeutically effective amount of a peptide according to the invention, more specifically an immunogenic form of a peptide according to the invention which is able to induce a T cell response effective against HPV, in particular a HLA class I-restricted $CD8^+$ cytotoxic T cell response.

Brief description of the drawings

Figure 1 gives the result of binding analyses of 240 HPV16 E6 and E7 nonapeptides to HLA-A2.1 expressed on 174CEM.T2 cells. Background fluorescence level (without adding peptides) was set on an arbitrary mean fluorescence level of 70. Binding of a peptide was regarded positive when twice the level of background fluorescence was reached. The 18 binding peptides are numbered 1 to 18; this numbering corresponds to the numbering in Table I.

Figure 2 gives the result of binding analyses of 247 HPV18 E6 and E7 nonapeptides to HLA-A2.1 expressed on 174CEM.T2 cells. Background fluorescence level (without adding peptides) was set on an arbitrary mean fluorescence level of 70. Binding of a peptide was regarded positive when twice the level of background fluorescence was reached. The 14 binding peptides are numbered 1 to 14; this numbering corresponds to the numbering in Table II.

Figure 3 is a graph showing the primary CTL response of human lymphocytes from healthy donor blood against an HPV16 peptide (peptide MLDLQPETT, No. 13 in Table I, SEQ ID NO 15). Bulk = bulk culture of CTL; clone = CTL clone from limiting dilution; irr. peptide = irrelevant peptide (control); and E/T ratio = effector target ratio.

Detailed description of the invention

The invention is directed to peptides comprising an amino acid sequence derived from a protein of HPV, wherein said amino acid sequence has the ability to bind to a human MHC Class I molecule. In view of our own experience with other viruses, the best candidates for induction of HLA class I restricted $CD8^+$ cytotoxic T cells are the strongest binding peptides.

A most preferred embodiment of the invention concerns peptides comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1. Specifically, such peptides comprise the following amino acid sequences derived from the noted regions of HPV16 (see Table I; the amino acids are identified by the one-letter code of amino acids).

TABLE I

Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A2.1

Peptide No.	Amino acid sequence	protein (region)	SEQ ID NO
-	AMFQDPQER	E6 (residues 7- 15)	1
1	KLPQLCTEL	E6 (residues 18 - 26)	2
2	QLCTELQTT	E6 (residues 21 - 29)	3
3	LCTELQTTI	E6 (residues 22 - 30)	4
4	ELQTTIHDI	E6 (residues 25 - 33)	5
5	LQTTIHDII	E6 (residues 26 - 34)	6
6	TIHDIILEC	E6 (residues 29 - 37)	7
7	IHDIILECV	E6 (residues 30 - 38)	8
8	CVYCKQQLL	E6 (residues 37 - 45)	9
-	FAFRDLCIV	E6 (residues 52- 60)	10
9	KISEYRHYC	E6 (residues 79 - 87)	11
10	PLCDLLIRC	E6 (residues 102-110)	12
11	TLHEYMLDL	E7 (residues 7 - 15)	13
12	YMLDLQPET	E7 (residues 11 - 19)	14
13	MLDLQPETT	E7 (residues 12 - 20)	15
14	RLCVQSTHV	E7 (residues 66 - 74)	16
15	TLEDLLMGT	E7 (residues 78 - 86)	17
16	LLMGTLGIV	E7 (residues 82 - 90)	18
17	GTLGIVCPI	E7 (residues 85 - 93)	19
18	TLGIVCPIC	E7 (residues 86 - 94)	20

Another most preferred embodiment of the invention concerns peptides comprising an amino acid sequence derived from protein E6 or E7 of HPV18, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1. Specifically, such peptides comprise the following amino acid sequences derived from the noted regions of HPV18 (see Table II; the amino acids are identified by the one-letter code of amino acids).

TABLE II

Peptides derived from HPV18 proteins E6 and E7 binding to HLA-A2.1

Peptide No.	Amino acid sequence	protein (region)	SEQ ID NO
1	KLPDLCTEL	E6 (residues 13 - 21)	21
2	SLQDIEITC	E6 (residues 24 - 32)	22
3	LQDIEITCV	E6 (residues 25 - 33)	23
4	EITCVYCKT	E6 (residues 29 - 37)	24
5	KTVLELTEV	E6 (residues 36 - 44)	25
6	ELTEVFEFA	E6 (residues 40 - 48)	26
7	FAFKDLFVV	E6 (residues 47 - 55)	27
8	DTLEKLTNT	E6 (residues 88 - 96)	28
9	LTNTGLYNL	E6 (residues 93 -101)	29
10	TLQDIVLHL	E7 (residues 7 - 15)	30
11	FQQLFLNTL	E7 (residues 86 - 94)	31
12	QLFLNTLSF	E7 (residues 88 - 96)	32
13	LFLNTLSFV	E7 (residues 89 - 97)	33
14	LSFVCPWCA	E7 (residues 94 -102)	34

Another preferred embodiment of the invention concerns peptides comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A1. Specifically, such peptides comprise the following amino acid sequences derived from the noted regions of HPV16 (see Table III; the amino acids are identified by the one-letter code of amino acids).

TABLE III

Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A1

Amino acid sequence	protein (region)	SEQ ID NO
YRDGNPYAV	E6 (residues 61- 69)	35
WTGRCMSCC	E6 (residues 139-147)	36
MSCCRSSRT	E6 (residues 144-152)	37
TTDLYCYEQ	E7 (residues 19- 27)	38
EIDGPAGQA	E7 (residues 37- 45)	39
HVDIRTLED	E7 (residues 73- 81)	40

Another preferred embodiment of the invention concerns peptides comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A3.2. Specifically, such peptides comprise the following amino acid sequences derived from the noted regions of HPV16 (see Table IV; the amino acids are identified by the one-letter code of amino acids).

TABLE IV

Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A3.2

Amino acid sequence	protein (region)	SEQ ID NO
AMFQDPQER	E6 (residues 7- 15)	1
IILECVYCK	E6 (residues 33- 41)	41
CVYCKQQLL	E6 (residues 37- 45)	9
VYCKQQLLR	E6 (residues 38- 46)	42
QQLLRREVY	E6 (residues 42- 50)	43
IVYRDGNPY	E6 (residues 59- 67)	44
YAVCDKCLK	E6 (residues 67- 75)	45
AVCDKCLKF	E6 (residues 68- 76)	46
VCDKCLKFY	E6 (residues 69- 77)	47
KFYSKISEY	E6 (residues 75- 83)	48
KISEYRHYC	E6 (residues 79- 87)	11
ISEYRHYCY	E6 (residues 80- 88)	49
RHYCYSLYG	E6 (residues 84- 92)	50
SLYGTTLEQ	E6 (residues 89- 97)	51
TTLEQQYNK	E6 (residues 93-101)	52
QQYNKPLCD	E6 (residues 97-105)	53
LIRCINCQK	E6 (residues 107-115)	54
HLDKKQRFH	E6 (residues 125-133)	55
CMSCCRSSR	E6 (residues 143-151)	56
SCCRSSRTR	E6 (residues 145-153)	57
CCRSSRTRR	E6 (residues 146-154)	58
HYNIVTFCC	E7 (residues 51- 59)	59
YNIVTFCCK	E7 (residues 52- 60)	60
CCKCDSTLR	E7 (residues 58- 66)	61
KCDSTLRLC	E7 (residues 60- 68)	62

Another preferred embodiment of the invention concerns peptides comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A11.2. Specifically, such peptides comprise the following amino acid sequences derived from the noted regions of HPV16 (see Table V; the amino acids are identified by the one-letter code of amino acids).

TABLE V

Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A11.2

Amino acid sequence	protein (region)	SEQ ID NO
AMFQDPQER	E6 (residues 7- 15)	1
IILECVYCK	E6 (residues 33- 41)	41
CVYCKQQLL	E6 (residues 37- 45)	9
VYCKQQLLR	E6 (residues 38- 46)	42
QQLLRREVY	E6 (residues 42- 50)	43
IVYRDGNPY	E6 (residues 59- 67)	44
YAVCDKCLK	E6 (residues 67- 75)	45
AVCDKCLKF	E6 (residues 68- 76)	46
VCDKCLKFY	E6 (residues 69- 77)	47
KISEYRHYC	E6 (residues 79- 87)	11
ISEYRHYCY	E6 (residues 80- 88)	49
LIRCINCQK	E6 (residues 107-115)	54
TGRCMSCCR	E6 (residues 140-148)	63
CMSCCRSSR	E6 (residues 143-151)	56
SCCRSSRTR	E6 (residues 145-153)	57
HYNIVTFCC	E7 (residues 51- 59)	59
YNIVTFCCK	E7 (residues 52- 60)	60
CCKCDSTLR	E7 (residues 58- 66)	61
VCPICSQKP	E7 (residues 90- 98)	64

Another preferred embodiment of the invention concerns peptides comprising an amino acid sequence derived from protein E6 or E7 of HPV16, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A24. Specifically, such peptides comprise the following amino acid sequences derived from the noted regions of HPV16 (see Table VI; the amino acids are identified by the one-letter code of amino acids).

TABLE VI

Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A24

Amino acid sequence	protein (region)	SEQ ID NO
MHQKRTAMF	E6 (residues 1- 9)	65
LQTTIHDII	E6 (residues 26- 34)	6
VYCKQQLLR	E6 (residues 38- 46)	42
LLRREVYDF	E6 (residues 44- 52)	66
VYDFAFRDL	E6 (residues 49- 57)	67
PYAVCDKCL	E6 (residues 66- 74)	68
KCLKFYSKI	E6 (residues 72- 80)	69
EYRHYCYSL	E6 (residues 82- 90)	70
HYCYSLYGT	E6 (residues 85- 93)	71
CYSLYGTTL	E6 (residues 87- 95)	72
RFHNIRGRW	E6 (residues 131-139)	73
RAHYNIVTF	E7 (residues 49- 57)	74

The data suggest that the peptides mentioned above are single polypeptides of identified sequences. However, homologs, isoforms or genetic variants of these peptides may exist within or outside the cellular environment. This invention encompasses all such homologs, isoforms or genetic variants of the above peptides provided that they bind to the HLA molecule in question.

Polypeptides that are homologs of the peptides specifically include those having amino acid sequences which are at least about 40% conserved in relation to the amino acid sequence set forth in

Tables I-VI, preferentially at least about 60% conserved, and more preferentially at least about 75% conserved.

It will be understood by one of ordinary skill in the art that other variants of the peptides shown above are included within the scope of the present invention. This particularly includes any variants that differ from the above mentioned and synthesized peptides only by conservative amino acid substitution. In particular, replacements of C (cysteine) by A (alanine), S (serine), α-aminobutyric acid and others are included as it is known that cysteine-containing peptides are susceptible to (air) oxidation during synthesis and handling. Many such conservative amino acid substitutions are set forth as sets by Taylor (1986).

Herein the peptides shown above or fragments thereof include any variation in the amino acid sequence, whether by conservative amino acid substitution, deletion, or other processes, provided that the polypeptides bind to the HLA molecule in question. The fragments of the peptides may be small peptides with sequences of as little as five or more amino acids, said sequences being those disclosed in Tables I-VI when said polypeptides bind to the HLA molecule in question.

Polypeptides larger than the peptides shown are especially included within the scope of the present invention when said polypeptides induce a HPV16- or HPV18-specific CTL response in appropriate individuals (e.g. HLA-A2.1 positive individuals in the case of HLA-A2.1 binding peptides) and include a (partial) amino acid sequence as set forth in Tables I-VI, or conservative substitutions thereof. Such polypeptides may have a length up to about 30 amino acids, preferably up to about 27 amino acids. Most preferably, however, the peptides have a length of from 9 to 12, more preferably 9 to 11 or even 9 to 10 amino acids, most of all preferably exactly 9 amino acids.

This invention includes the use of polypeptides generated by every means, whether genetic engineering, peptide synthesis with solid phase techniques or others. The foregoing peptides may have various chemical modifications made at the terminal ends and still be within the scope the present invention. Also other chemical

modifications are possible, particularly cyclic and dimeric configurations. The term "derivatives" intends to cover all such modified peptides.

The polypeptides of the present invention find utility for the treatment or prevention of diseases involving HPV16 or HPV18 such as genital warts, cervical cancer or others that are linked to HPV16 or HPV18.

For all applications the peptides are administered in an immunogenic form. Since the peptides are relatively short, this may necessitate conjugation with an immunogenicity conferring carrier material such as lipids or others or the use of adjuvants.

The magnitude of a prophylactic or a therapeutic dose of polypeptides of this invention will, of course, vary with the group of patients (age, sex, weight, etcetera), the nature of the severity of the condition to be treated, the particular polypeptide of this invention and its route of administration. Any suitable route of administration may be employed to achieve an effective dosage of a polypeptide identified by this invention, as well as any dosage form well known in the art of pharmacy. In addition the polypeptides may also be administered by controlled release means and/or delivery devices. They may also be administered in combination with other active substances, such as, in particular, T-cell activating agents like interleukine-2 etc.

The peptides of this invention may also be useful for other purposes, such as diagnostic use. For example, they may be used to check whether a vaccination with a peptide according to the invention has been successful. This may be done in vitro by testing whether said peptide is able to activate T cells of the vaccinated person.

The following examples illustrate the present invention without limiting the same thereto.

EXAMPLE 1

Materials

Peptide synthesizer:

Abimed AMS 422 (Abimed Analysen-Technik GmbH, Langenfeld, Germany).

Synthesis polymer:

Tentagel S AC (0.17-0.24 meq/g, Rapp Polymere, Tübingen, Germany).

HPLC equipment:

The HPLC system used for analysis and purification of peptides consisted of: autosampler 2157, HPLC pump 2248, variable wavelength monitor VWM 2141, column oven 2155, low pressure mixer, all of Pharmacia Nederland B.V., Woerden, The Netherlands, a Star LC-20 dot matrix printer, Star Micronics Co., Ltd., all parts controlled by a Tandon PCAs1/386sx computer, Tandon Computer Benelux B.V., Amsterdam, The Netherlands.

Lyophylizer:

Virtis Centry, The Virtis Company, Inc., Gardiner (NY), USA, equipped with an Alcatel 350C vacuumpump, Alcatel CIT, Malakoff, France, connected to a Christ Alpha RVC vacuo-spin, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany.

Centrifuge:

MSE Mistral 6L, Beun de Ronde, Abcoude, The Netherlands.

Mass spectrometer:

Bioion plasma desorption mass spectrometer (PDMS), Applied Biosystems, Inc., Foster City (CA), USA.

Amino acid Analysis:

HP Aminoquant, Hewlett Packard, Amstelveen, The Netherlands.

Chemicals:

All chemicals were used without further purification unless stated otherwise.

Fmoc (9-fluorenylmethyloxycarbonyl) amino acids were of the L-configuration, bearing the following side chain protecting groups: t-Bu (tert-butyl) for Asp, Glu, Tyr, Ser and Thr; Trt (trityl) for His, Asn and Gln; Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl) for Arg; Boc (tert-butyloxycarbonyl) for Lys, all

Novasyn and purchased from Pharmacia Nederland B.V., Woerden, The Netherlands.

Piperidine was purchased from Aldrich Chemie Benelux N.V., Brussels, Belgium.

BOP (benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate) was obtained from Richelieu Biotechnologies, St-Hyacinthe, Canada.

N-methylmorpholin (NMM, Janssen Chimica, Tilburg, The Netherlands) was distilled from NaOH at atmospheric pressure before use.

N-methylpyrrolidone (NMP, Aldrich Chemie) was vacuum-distilled under a nitrogen atmosphere (b.p. 78-80°C, 18 mm Hg) before use.

Acetonitrile (HPLC-grade) was purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland.

Ether (Baker Analyzed grade), pentane (Baker grade) and acetic acid (Baker Analyzed grade) were purchased from J.T. Baker B.V., Deventer, The Netherlands.

Ethanethiol was obtained from Fluka Chemie, Brussels, Belgium.

Dichloromethane and N,N-dimethylacetamide (DMA) were purchased from Janssen Chimica, Tilburg, The Netherlands.

Trifluoroacetic acid (TFA, z.S. grade) was obtained from Merck-Schuchardt, Hohenbrunn, Germany.

Disposables:

Polypropylene reaction vessels containing a PTFE filter were purchased from Abimed Analysen-Technik GmbH, Langenfeld, Germany.

All other disposables used were made of polypropylene and obtained from Sarstedt B.V., Etten-Leur, The Netherlands.

Experimental conditions:

All experiments were performed at room temperature unless stated otherwise. All Fmoc protected aminoacids, synthesis polymers, peptides and TFA were stored at -20°C.

Peptide synthesis

Peptides were synthesized by solid phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) (see Gausepohl and Frank, 1990; Gausepohl et al., 1990).

The peptides were made in various runs, in each of which 48 different peptides were synthesized simultaneously.

Tentagel S AC (Rapp et al., 1990; Sheppard and Williams, 1982), a graft polymer of polyethyleneglycol spacer arms on a polystyrene matrix, was used as a resin (40-60 mg per peptide, 10 µmol Fmoc amino acid loading).

Repetitive couplings were performed by adding a mixture of 90 µl 0.67 M BOP (Gausepohl et al., 1988; Castro et al., 1975) in NMP, 20 µl NMM in NMP 2/1 (v/v) and 100 µl of an 0.60 M solution of the appropriate Fmoc amino acid (Fields and Noble, 1990) in NMP (6-fold excess) to each reaction vessel. At 70% of the reaction time approximately 50 µl dichloromethane was added to each reaction vessel.

Fmoc-deprotection was performed by adding 3 times 0.8 ml of piperidine/DMA 1/4 (v/v) to each reaction vessel.

Coupling- and deprotection times were increased as the synthesis proceeded, starting with 30 min and 3 times 3 min respectively.

Washings after couplings and Fmoc-deprotections were done with 6 times 1.2 ml DMA. After the required sequence had been reached and the last Fmoc-protection was removed the peptidylresin was washed extensively with DMA, dichloromethane, dichloromethane/ether 1/1 (v/v) and ether respectively, and dried.

Peptide cleavage and isolation

Cleavage of the peptides from the resin and removal of the side chain protecting groups was performed by adding 6 times 200 µl TFA/water 19/1 (v/v) at 5 min intervals to each reaction vessel, thus yielding free carboxylic peptides. For Trp-containing peptides TFA/water/ethanethiol 18/1/1 (v/v/v) was used.

Two hours after the first TFA addition the peptides were precipitated from the combined filtrates by addition of 10 ml

ether/pentane 1/1 (v/v) and cooling to -20°C. The peptides were isolated by centrifugation (-20°C, 2500g, 10 min).

After treatment of the pellet with ether/pentane 1/1 (v/v) and isolation by the same centrifugation procedure, the peptides were dried at 45°C for 15 min.

Each of the peptides was dissolved in 2 ml water (or 2 ml 10 vol.% acetic acid), the solution frozen in liquid nitrogen for 3 min, and lyophylized while being centrifuged (1300 rpm, 8-16 h).

Analysis and purification

The purity of the peptides was determined by reversed phase HPLC; an aliquot of about 50 nmol was dissolved in 100 µl 30 vol.% acetic acid. Of this solution 30 µl was applied to an RP-HPLC system equipped with a ternary solvent system; A: water, B: acetonitrile, C: 2 vol.% TFA in water.

Gradient elution (1.0 ml/min) was performed from 90% A, 5% B, 5% C to 20% A, 75% B, 5%C in 30 min. Detection was at 214 nm.

Samples taken at random were analysed by mass spectrometry on a PDMS. The 31 binding peptides were all analysed by mass spectrometry on a PDMS and by quantative amino acid analysis after hydrolysis on a HP Aminoquant. Of all analysed samples the difference between calculated and measured masses was within the experimental error (0.1%) as specified by the producer of the equipment used. All aminoacid compositions were as expected.

EXAMPLE 2

Peptides

Of all 240 HPV16 peptides and 247 HPV18 peptides that had been freeze dried, 5 mg was weighed and dissolved in 1 ml of distilled water adjusted with 5N NaOH to a pH of 12. Peptides that did not readily dissolve were treated with 150 µl of 100% acetic acid glacial (CH_3COOH, Merck Darmstadt, Germany: 56-1000) after which the pH was neutralized to pH7 with 5N NaOH diluted in distilled water (Merck Darmstadt, Germany: 6498). Peptides which still did not dissolve were treated with 100 µl of 5N NaOH to pH 12

after which the pH was neutralized to pH 7 with 10% acetic acid glacial in distilled water. Of all peptides a dilution of 1 mg/ml in 0.9% NaCl was made.

Cells

174CEM.T2 cells were cultured in Iscove's modified Dulbecco's medium (Biochrom KG Seromed Berlin, Germany: F0465) supplemented with 100IU/ml penicillin (Biocades Pharma, Leiderdorp, The Netherlands), 100 µg/ml kanamycin (Sigma St. Louis, USA:K-0254), 2mM glutamine (ICN Biomedicals Inc. Costa Mesa, CA, USA:15-801-55) and 10% fetal calf serum (FCS, Hyclone Laboratories Inc. Logan, Utah, USA:A-1115-L). Cells were cultured at a density of 2.5 x 10^5/ml during 3 days at 37°C, 5% CO_2 in humified air.

Peptide binding

174CEM.T2 cells were washed twice in culture medium without FCS and put in serum-free culture medium to a density of 2 x 10^6 cells/ml. Of this suspension 40 µl was put into a V bottomed 96 well plate (Greiner GmbH, Frickenhausen, Germany: 651101) together with 10 µl of the individual peptide dilutions (of 1 mg/ml). The end concentration is 200 µg/ml peptide with 8 x 10^4 174CEM.T2 cells. This solution was gently agitated for 3 minutes after which an incubation time of 16 hours at 37°C, 5% CO_2 in humified air took place. Then cells were washed once with 100 µl 0.9% NaCl, 0.5% bovine serum albumin (Sigma St. Louis, USA: A-7409), 0.02% NaN_3 (Merck Darmstadt, Germany:822335). After a centrifuge round of 1200 rpm the pellet was resuspended in 50 µl of saturating amounts of HLA-A2.1 specific mouse monoclonal antibody BB7.2 for 30 minutes at 4°C. Then cells were washed twice and incubated for 30 minutes with $F(ab)_2$ fragments of goat anti-mouse IgG that had been conjugated with fluoresceine isothiocyanate (Tago Inc Burlingame, CA, USA: 4350) in a dilution of 1:40 and a total volume of 25 µl.

After the last incubation, cells were washed twice and fluorescence was measured at 488 nanometer on a FACScan flow-cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The results

are shown in fig. 1 (HPV16 peptides) and fig. 2 (HPV18 peptides), respectively.

The 174CEM.T2 cell line expresses "empty" and unstable HLA-A2.1 molecules that can be stabilized when a peptide is binding to the peptide presenting groove of these molecules. A stabilized HLA-A2.1 molecule that will not easily degrade is the result of binding of an analyzed peptide. This leads to an increase in cell surface expression of the HLA-A2.1 molecule.

Results

In order to identify E6 and E7 region peptides of HPV16 and HPV18 that could bind to HLA-A2.1 molecules expressed by 174CEM.T2 cells, the amino acid sequences of E6 and E7 of HPV16 and HPV18 were examined (4). Every amino acid in the E6 and E7 region was used as the first amino acid of a 9 amino acid long peptide. In this way the entire E6 and E7 regions of HPV16 and HPV18 were covered. Nine amino acid long peptides were chosen because they fit the presently known rules for length of peptides that bind to the groove of HLA-A2.1 molecules (reviewed by Kast and Melief, 1991). For practical reasons, in a first series of experiments alanine residues were used in the tested peptides instead of the cysteine residues occurring in the natural sequence. Thereafter, a second series of experiments was carried out with peptides containing the cysteine residues of the natural sequence.

Only the peptides Nos. 1-18 of Table I and Nos. 1-14 of Table II (including those containing alanine residues instead of the cysteine residues) were able to significantly upregulate the expression of HLA-A2.1 molecules measured as mean HLA-A2.1 fluorescence of 174CEM.T2 cells indicating their binding to the HLA-A2.1 molecule as described in Example 2. None of the 222 + 233 other peptides were able to do this. The results of the fluorescence measurement are given in Tables VII and VIII and shown in Figures 1 and 2. The peptides are numbered in accordance with the numbering in Tables I and II.

MF = Mean Fluorescence

$$FI = \text{Fluorescence Index} = \frac{(MF)_{experiment} - (MF)_{blank}}{(MF)_{blank}}$$

With background fluorescence level (without adding peptides) set on a Fluorescence Index level of 0, binding of a peptide was regarded positive when the level of fluorescence was ≥ 0.5.

TABLE VII: HPV16 peptides

Peptide	FI	MF
1	1.6	168.6
2	2.2	211.9
3	0.7	113.6
4	0.6	108.0
5	1.6	170.4
6	1.8	184.5
7	1.3	150.7
8	2.0	195.0
9	2.2	211.7
10	2.1	204.8
11	1.2	144.5
12	3.3	283.9
13	2.3	217.6
14	2.8	250.0
15	1.8	182.8
16	0.8	118.8
17	2.3	216.1
18	2.5	227.8

TABLE VIII: HPV18 peptides

Peptide	FI	MF
1	2.1	439.3
2	0.7	243.9
3	1.3	327.8
4	0.5	206.0
5	2.3	467.5
6	1.1	289.0
7	2.3	462.9
8	0.7	238.2
9	1.5	352.5
10	2.7	519.6
11	0.8	244.5
12	0.8	258.2
13	1.6	360.3
14	0.9	262.0

These experiments indicate that only a limited proportion of peptides have the ability to bind to the HLA-A2.1 molecule and are therefore the only candidates of the HPV16 and HPV18 E regions to be recognized by human CTL because CTL recognize peptides only when bound to HLA molecules.

EXAMPLE 3

This example illustrates in vitro induction of primary immune response against HPV peptides using the processing defective cell line 174CEM.T2.

The expression of HLA-A2.1 on 174CEM.T2 cells (T2) is increased by incubating T2 cells in medium containing relevant peptide. T2 cells will present the relevant peptide bound to HLA-A2.1 in high amount and therefore are good antigen presenting cells (APC). In the response inducing

method described below the T2 cell line is used as APC and post-Ficoll mononuclear cells are used as responder cells.

Method

1) Peptide loading of HLA-A2.1 on T2

T2 cells in a concentration of 2 x 10^6 cells per ml were incubated for 13 h at 37°C in a T 25 flask (Becton Dickinson, Falcon, Plymouth Engeland cat.nr. 3013) in serum-free IMDM (= Iscoves Modified Dulbecco's Medium: Biochrom KG, Seromed Berlin, Germany, cat.nr. F0465) with glutamine (2mM, ICN Biochemicals Inc., Costa Meisa, USA, cat.nr. 15-801-55), antibiotics (100 IU/ml penicilline (Brocades Pharma, Leiderdorp, The Netherlands, 100 µg/ml kanamycine (Sigma, St. Louis, USA, K-0245)) and the selected peptide MLDLQPETT (= JWK3; SEQ ID NO:15) in a concentration of 80 µg/ml.

2) Mitomycine C treatment of T2 (APC)

These incubated T2 cells were spun down and subsequently treated in a density of 20 x 10^6 cells/ml with Mitomycine C (50µg/ml) in serum-free RPMI (Gibco Paislan, Scotland, cat.nr. 041-02409) medium for 1 h at 37°C. Hereafter the T2 cells were washed three times in RPMI.

3) Preparing for primary immune response induction

All wells of a 96-well-U-bottom plate (Costar, Cambridge, USA, cat.nr. 3799) were filled with 100,000 Mitomycine C-treated T2 cells in 50µl serum-free, complete RPMI medium (glutamine (2mM, ICN Biochemicals Inc., Costa Meisa, USA, cat.nr. 15-801-55), penicilline (100 IU/ml, Brocades Pharma, Leiderdorp, The Netherlands), kanamycine (100µg/ml, Sigma, St. Louis, USA, K-0245)) and the peptide MLDLQPETT in a concentration of 80 µg/ml.

4) Responder cells

Responder cells are mononuclear peripheral blood lymphocytes (PBL) of a HLA-A2.1 subtyped donor (= C.B.). The

PBL were separated from a buffy coat by Ficoll-procedure (Ficoll preparation: Lymphoprep of Nycomed-pharma, Oslo, Norway, cat.nr. 105033) and washed two times in RPMI. After separation and washing, the PBL were resuspended in complete RPMI medium with 30% human pooled serum (HPS) (HPS is tested for suppression activity in Mixed Lymphocyte Cultures).

5) Incubation of primary immune response

400,000 PBL-C.B. in 50µl of medium (the medium described in header 4) were added to each well of the 96-well-U-bottom plate already filled with T2 cells and cultured for 7 days at 37°C in an incubator with 5% CO_2 and 90% humidity.

6) Restimulation (day 7)

On day 7 after incubation of PBL, peptide MLDLQPETT and T2 cells (headers 1-5), the PBL-C.B. were restimulated with peptide MLDLQPETT. For this purpose all cells and medium out of the 96 wells were harvested. Viable cells were isolated by Ficoll-procedure and washed in RPMI. In a new 96-well-U-bottom plate 50,000 of these viable cells were seeded to each well together with 50µl complete RPMI medium with 15% HPS. Per well 20,000 autologous, irradiated (3000 rad) PBL and 50,000 autologous, irradiated (10000 rad) EBV-transformed B-lymphocytes (= EBV-C.B.) were added together with 50µl of complete RPMI medium with 15% HPS and peptide MLDLQPETT in a concentration of 80µg/ml. The cells were cultured for 7 days at 37°C in an incubator with 5% CO_2 and 90% humidity.

7) Restimulation (day 14)

On day 14 after incubation of PBL, peptide MLDLQPETT and T2 cells (headers 1-5), the PBL-C.B.were restimulated with peptide MLDLQPETT. To do so the procedure under header 6 is repeated.

8) Cloning by Limiting Dilution

On day 21 after incubation of PBL, peptide MLDLQPETT and T2 cells, cells and medium out of the 96 wells were harvested. Viable cells were isolated by Ficoll-procedure and washed in complete RPMI with 15% HPS. This bulk of viable cells was cloned by Limiting Dilution. Into each well of a new 96-well-U-bottom plate (Costar, Cambridge, USA, cat. nr. 3799) 50µl complete RPMI medium with 15 % HPS was added together with 100 viable cells (= HPV16 bulk anti MLDLQPETT). For other new 96-well-U-bottom plates this was exactly repeated except for the number of cells for wells: subsequent plates contained 10, 1, or 0.3 cells per well. To all wells 20,000 pooled and irradiated (3000 rad) PBL of four different donors and 10,000 pooled and irradiated (10,000 rad) EBV-transformed B-cells of three different HLA-A2.1 donors (VU-4/518/JY) were added together with 50µl of complete RPMI medium with 15% HPS and peptide MLDLQPETT in a concentration of 40µg/ml, Leucoagglutinin in a concentration of 2% (Pharmacia, Uppsala, Sweden, cat.nr. 17-063-01), human recombinant IL-2 in a concentration of 120 IU/ml (Eurocetus, Amsterdam, The Netherlands).

9) Expand clones

Add per well, in a final volume of 100 µl =>

- 25,000 viable cells
- 20,000 irradiated PBL-pool (as in header 8)
- 10,000 irradiated EBV-pool (as in header 8)
- 2 µg peptide MLDLQPETT
- 6 IU recombinant IL-2.

On day 49 a cytotoxicity assay was performed with 65 clones and one bulk as effector cells and T2 (with or without the relevant peptide MLDLQPETT) as target cells. Background killing is defined as killing of T2 cells incubated with an irrelevant (but HLA-A2.1 binding) peptide: ATELQTTIH.

The HPV16 bulk (C.B.) anti MLDLQPETT seemed to be specific for killing MLDLQPETT-sensitized T2 cells. All the clones were not specific.

A new limiting dilution was done with the HPV16 bulk (C.B.) anti MLDLQPETT cells (as in header 8+9).

On day 28 after the new limiting dilution a cytotoxicity assay was performed with five clones (ID10, ID12, ID19, ID26, ID92) and one bulk. A representative clone is shown in fig. 3.

EXAMPLE 4

This example illustrates an immunochemical peptide-MHC binding assay which was used to determine which HPV16 protein E6 and E7 peptides bind to HLA-A1, A2.1, A3.2, A11.2 and A24 molecules.

The method utilizes purified class I molecules and radiolabeled synthetic probes based on consensus peptides. Competitor peptides tested for their binding to the class I molecules compete for this binding with the radiolabeled consensus peptides. The HLA-A1, A2.1, A3.2, A11.2, and A24 molecules were isolated from the following cell lines, respectively: the EBV (Eppstein Barr Virus) transformed cell line Steinlin, the EBV transformed cell line JY, the EBV transformed cell line GM3107, the cell line BVR and the EBV transformed cell line KT3. After large scale culture, cells were lysed in NP40 (Fluka Biochemika, Buchs, Switzerland) and the lysate was passed over two pre-columns of inactivated sepharose CL 4B and Prot A Sepharose. Class I molecules were then purified from the cell lysate by affinity chromatography using Prot A Sepharose beads conjugated with B2.23.2 (anti-HLA-B and HLA-C) and anti-human HLA. The lysate was first depleted of B and C molecules by repeated passage over the B2.23.2 column. Remaining HLA-A molecules were then captured by the W6/32 column and eluted by pH 11.5 DEA/1% OG neutralized with 1 mM Tris pH 6.8 and concentrated by ultrafiltration on Amicon 30 K_D cartridges.

For binding assays, MHC amounts that resulted in binding of 15% of the radiolabeled synthetic probes (normally in the 10-50 nM range) were incubated in 0.05% NP40-PBS with about 5 nM of radiolabeled peptides and titrated amounts of unlabeled competitor peptides to be tested (usually in the 10 mg to 1 ng/ml range) in the presence of 1 μM β2M and a cocktail of protease inhibitors (with final concentrations of 1 mM PMSF, 1.3 mM 1,10-Phenanthroline, 73 μM Pepstatin A, 8 mM EDTA, 200 μM N-α-p-tosyl-L-Lysine Chloromethyl ketone). After two days at 23°C the percent of MHC-bound radioactivity was measured by size exclusion chromatography on a TSK2000 gel filtration as described by Sette et al. (1992).

Probe peptides were iodinated by using the Chloramine T method described by Buus et al. (1987). The sequences of the probe peptides used for the aforementioned HLA molecules were YLEPAIAKY for A1, FLPSDYFPSV for A2.1, KVFPYALINK for A3.2, AVDLYHFLK for A11.2, and AYIDNYNKF for A24.

To allow comparison of the data obtained in different experiments, a relative binding figure was calculated for each peptide by dividing the 50% inhibition dose (IC_{50}) for the positive control for inhibition of unlabeled probe peptides by the 50% inhibition doses for each tested peptide. The values of the 50% inhibition dose for the probes was: 81 nM for A1, 5 nM for A2.1, 30 nM for A3.2, 9 nM for A11.2 and 22 nM for A24.

Each competitor peptide was tested in two to four completely independent experiments. Since cysteine containing peptides are susceptible to (air) oxidation during synthesis and handling, these peptides were synthesized with an alanine instead of a cysteine. Arbitrarily, the competitor peptides were categorized as good binders, intermediate binders, weak binders and negative binders when they fell into the following ratio categories: 1.0-0.1, 0.1-0.01, 0.01-0.001, and < 0.001, respectively.

The results are shown in Tables IX to XIII.

TABLE IX: HPV16 E6 and E7 peptides binding to HLA-A1 in immunochemical assay

Peptide	protein (region)	SEQ ID NO	binding ratio to standard#
YRDGNPYAV	E6 (residues 61- 69)	35	0.008
WTGRCMSCC	E6 (residues 139-147)	36	0.020
MSCCRSSRT	E6 (residues 144-152)	37	0.019
TTDLYCYEQ	E7 (residues 19- 27)	38	0.023
EIDGPAGQA	E7 (residues 37- 45)	39	0.025
HVDIRTLED	E7 (residues 73- 81)	40	0.014

The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 81 ±30 nM.

TABLE X: Additional HPV16 E6 and E7 peptides binding to HLA-A2.1 in immunochemical assay

Peptide	protein (region)	SEQ ID NO	binding ratio to standard#
AMFQDPQER	E6 (residues 7- 15)	1	0.0033
FAFRDLCIV	E6 (residues 52- 60)	10	0.3700

The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 6 ±1 nM.

TABLE XI: HPV16 E6 and E7 peptides binding to HLA-A3.2 in immunochemical assay

Peptide	protein (region)	SEQ ID NO	binding ratio to standard#
AMFQDPQER	E6 (residues 7- 15)	1	0.1000
IILECVYCK	E6 (residues 33- 41)	41	1.5000
CVYCKQQLL	E6 (residues 37- 45)	9	0.0320
VYCKQQLLR	E6 (residues 38- 46)	42	0.0012
QQLLRREVY	E6 (residues 42- 50)	43	0.0058
IVYRDGNPY	E6 (residues 59- 67)	44	3.0000
YAVCDKCLK	E6 (residues 67- 75)	45	0.0012
AVCDKCLKF	E6 (residues 68- 76)	46	0.0056
VCDKCLKFY	E6 (residues 69- 77)	47	0.0025
KFYSKISEY	E6 (residues 75- 83)	48	0.0100
KISEYRHYC	E6 (residues 79- 87)	11	0.0044
ISEYRHYCY	E6 (residues 80- 88)	49	0.0064
RHYCYSLYG	E6 (residues 84- 92)	50	0.0036
SLYGTTLEQ	E6 (residues 89- 97)	51	0.0080
TTLEQQYNK	E6 (residues 93-101)	52	0.0780
QQYNKPLCD	E6 (residues 97-105)	53	0.0045
LIRCINCQK	E6 (residues 107-115)	54	3.7000
HLDKKQRFH	E6 (residues 125-133)	55	0.4400
CMSCCRSSR	E6 (residues 143-151)	56	0.1800
SCCRSSRTR	E6 (residues 145-153)	57	0.0200
CCRSSRTRR	E6 (residues 146-154)	58	0.0020
HYNIVTFCC	E7 (residues 51- 59)	59	0.0260
YNIVTFCCK	E7 (residues 52- 60)	60	0.0067
CCKCDSTLR	E7 (residues 58- 66)	61	0.0016
KCDSTLRLC	E7 (residues 60- 68)	62	0.0012

The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 30 ±3 nM.

TABLE XII: HPV16 E6 and E7 peptides binding to HLA-A11.2 in immunochemical assay

Peptide	protein (region)	SEQ ID NO	binding ratio to standard#
AMFQDPQER	E6 (residues 7- 15)	1	0.8400
IILECVYCK	E6 (residues 33- 41)	41	6.7000
CVYCKQQLL	E6 (residues 37- 45)	9	0.0450
VYCKQQLLR	E6 (residues 38- 46)	42	0.0022
QQLLRREVY	E6 (residues 42- 50)	43	0.0084
IVYRDGNPY	E6 (residues 59- 67)	44	0.4700
YAVCDKCLK	E6 (residues 67- 75)	45	0.0074
AVCDKCLKF	E6 (residues 68- 76)	46	0.0037
VCDKCLKFY	E6 (residues 69- 77)	47	0.0030
KISEYRHYC	E6 (residues 79- 87)	11	0.0076
ISEYRHYCY	E6 (residues 80- 88)	49	0.4300
LIRCINCQK	E6 (residues 107-115)	54	0.0120
TGRCMSCCR	E6 (residues 140-148)	63	0.0012
CMSCCRSSR	E6 (residues 143-151)	56	0.0084
SCCRSSRTR	E6 (residues 145-153)	57	0.0330
HYNIVTFCC	E7 (residues 51- 59)	59	0.0010
YNIVTFCCK	E7 (residues 52- 60)	60	0.0060
CCKCDSTLR	E7 (residues 58- 66)	61	0.0110
VCPICSQKP	E7 (residues 90- 98)	64	0.0012

The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 10 ±3 nM.

TABLE XIII: HPV16 E6 and E7 peptides binding to HLA-A24 in immunochemical assay

Peptide	protein (region)	SEQ ID NO	binding ratio to standard#
MHQKRTAMF	E6 (residues 1- 9)	65	0.0049
LQTTIHDII	E6 (residues 26- 34)	6	0.0200
VYCKQQLLR	E6 (residues 38- 46)	42	0.0011
LLRREVYDF	E6 (residues 44- 52)	66	0.0023
VYDFAFRDL	E6 (residues 49- 57)	67	0.0610
PYAVCDKCL	E6 (residues 66- 74)	68	0.0055
KCLKFYSKI	E6 (residues 72- 80)	69	0.1100
EYRHYCYSL	E6 (residues 82- 90)	70	0.0460
HYCYSLYGT	E6 (residues 85- 93)	71	0.0037
CYSLYGTTL	E6 (residues 87- 95)	72	0.1200
RFHNIRGRW	E6 (residues 131-139)	73	0.1000
RAHYNIVTF	E7 (residues 49- 57)	74	0.0670

The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 22 ±6 nM.

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